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14. ABSTRACT

Radiotherapy is generally considered to be immunosuppressive, whereas we hypothesized that it modulates immune responses and has profound effects on the immune system rather than eliminating of lymphocytes. The goal of this study was to determine how radiation affects the presentation of prostate specific antigen (PSA), to investigate new potential mechanisms of altered immune function after radiation therapy, and to devise strategies to overcome radiation-induced immunosuppression in prostate cancer using treatment with IL-3 and/or GM-CSF. In addition, because of the high risk nature of the experiments and the high PSA expression levels in prostate cancer patients that might interfere with its efficacy, we developed a second model using survivin as an antigen, since it also is over-expressed in prostate and other cancers.

15. SUBJECT TERMS
Radiation, Dendritic Cells, Cytokines, PSA

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Introduction

Killing of radiosensitive lymphocytes is usually considered the main effect of ionizing radiation on the immune system. However, radiation is more than a cytotoxic agent. Our recent study has shown that radiation modulates the immune system by affecting dendritic cell (DC) function. We proposed that radiation-induced immune suppression could result from proteasome inhibition and/or expression of immunosuppressive cytokines and related molecules by DCs. We hypothesized that this form of radiation-induced immunosuppression might be circumvented using cytokines such as IL-3 and/or GM-CSF as an immune booster. In this study, we developed a system for examining the effects of radiation on presentation of prostate specific antigen (PSA) by DCs. We sought to uncover possible mechanisms by which radiation might cause alterations in antigen presentation. We developed strategies to deliver IL-3 in combination with PSA in attempts to alter DC antigen presenting function. The final goal in this study is to translate radiation-induced tumor cell death into generation of tumor immunity in the hope of optimizing therapy for localized and disseminated prostate cancer.

Body

In the first series of experiments, we attempted to develop systems for studying the effects of radiation on presentation of PSA by DCs. To investigate the endogenous pathway of antigen presentation, we first made adenoviral vectors expressing PSA (AdV-PSA) and used them to deliver PSA to DC and to immunize C57BL/6 mice. The DCs were irradiated (10 Gy) or non-irradiated before immunization. We were able to measure responses in the spleens of immunized mice as assessed by IFN-gamma and IL-4 producing cells in an ELISPOT assay (Fig. 2 in 2006 annual report). Irradiation of DCs just before using them for immunization slightly decreased the number of lymphocytes expressing IFN-gamma and IL-4. This proved that we could develop a model system for examining immune responses to PSA. Unfortunately, this model is not very relevant to humans because the PSA epitopes recognized by C57BL/6 mice are unknown but different from those recognized by humans. We therefore attempted to translate the initial findings into a humanized HLA-2.1/Kb mouse model, where we would know the epitopes that might be presented. In this experiment, mice were immunized in the same way and spleen cells were stimulated in vitro with the immunodominant PSA epitope PSA-3 prior to ELISPOT assay. A similar significant inhibitory effect of radiation on DC presentation of PSA was observed (Fig. 1).

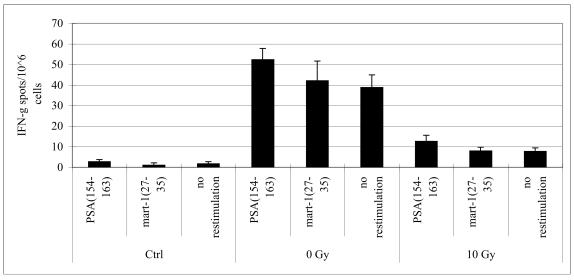

Fig. 1: Radiation effects on PSA processing by humanized HLA-A2/Kb DCs. HLA-A2.1 mice were injected with DCs treated with or without 10 Gy and with AdVPSA. Spleens were harvested 10-14 days after DC immunization and restimulated with either PSA-3 or non-specific peptide MART-1 (27-35) or no stimulation. The production of IFN-gamma was assessed by ELISPOT.

We also performed experiments designed to determine if antigen processing or antigen presentation was the target of the radiation effect. In the contrast to Fig 1, irradiation of DC enhanced their ability to present PSA-3 when it was added to them exogenously in the form of peptide. The number of IFN-gamma and IL-4 producing lymphocytes increased significantly in 10 Gy treated peptide-pulsed DC (Fig. 3 in 2006 annual report). These findings clearly indicate that radiation suppresses the ability of DC to process endogenous PSA but enhances their ability to present exogenous PSA-peptide. Therefore, the radiation-induced defect is in the production of immunogenic peptide from the whole PSA molecule, which is performed by the proteasome, or in its transport to the surface for presentation, while presentation of peptide might be enhanced by DC maturation, increased stability of MHC/peptide complexes on the surface of the cell, or by radiation-induced alterations in production of co-accessory molecules or cytokines.

In extensive series of many experiments we attempted to tease out the mechanisms underlying the effects of radiation on DC function. Our data showed that (1) radiation rapidly inhibits proteasome function in DCs; (2) radiation stabilizes peptide binding on MHC class I molecules at least for 24 hr (Fig. 4 in 2006 annual report); (3) irradiated DCs are inferior stimulators in a mixed lymphocyte reaction (MLR) (Fig. 1 in 2006 annual report); (4) irradiated DC introduced a tolerogenic signal for the immune system (Fig. 5 in 2004 annual report; Liao et al.); and (5) immunosuppressive cytokines, IL-10 and TGF-beta (Fig. 2 in 2004 annual report), were not enhanced after 10 Gy. However, the expression of TNFR I and II was increased following irradiation (Fig. 6 and 7 in 2006 annual report).

TNFRs can be shed from the cell surface constitutively but this can also be induced during immune responses. This mechanism is thought to limit innate immune responses and may be important for Th1 polarization. In an adaptive immune setting, a neutralizing antibody to TNFR has been shown to inhibit IL-12 release by DCs and, as a consequence, IFN-gamma production by T cells during an allogenic MLR (Becher et al). Since TNFR II engagement is important for co-stimulation of T cells, by releasing TNFR, DCs may limit the extent of vital co-stimulation of T cells to which antigen they are presenting. We were therefore following up our findings on radiation-induced shedding of TNFRs by DCs as a possible mechanism for their diminished ability to induce T cell priming in vivo. We tested this hypothesis using knockout mice, TNFR I (p55-/-), TNFR II (p75-/-) and double knockout (p55-/-p75-/-) on the C57/BL6 background.

We had some problems with our expression systems and chose to use a MART antigen as a model to initially examine the effects of TNFR deletion. It generally shows identical behavior though even here the effect of radiation was not marked. However, in repeated experiments, IFN-gamma expressing T cell responses were increased in p55-/- knockout mice after 10 Gy treatment of DCs that were endogenously expressing antigen, but the opposite effect was observed in p75-/- knockout mice. We concluded that TNF receptor I (p55) probably act as a “brake” on immunity in this system (Fig. 2 and Fig. 2 in 2006 annual report). These experiments are being repeated as we now have our immune monitoring for PSA-specific responses optimized.

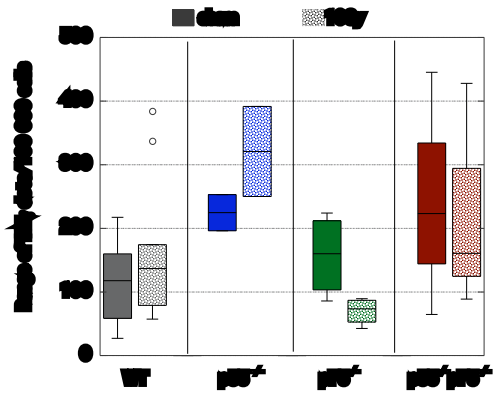

Fig. 2: Radiation differentially modulates antigen presentation by dendritic cells lacking the receptors for TNF. Dendritic cells derived from bone marrow cultures of p55-/- and 75-/- single or double knockout mice were irradiated with 10 Gy or left untreated prior to transduction with AdVMART1 and used for s.c. immunization. IFN-gamma production by activated splenocytes was measured by ELISPOT assays as above. Data are shown as the range of IFN-gamma spots/10^5 splenocytes re-stimulated with EL4-MART1 of three independent experiments.

Finally, we followed up on our observation that irradiation of DCs might generate cells capable of inducing immune tolerance. One mechanism for this effect could be the generation of CD4+CD25+Foxp3+ regulatory T cells (Treg) that would inhibit the generation of T effector cells. We used a TRAMP C1 prostate tumor model to examine the feasibility of this hypothesis. As shown in Fig. 3, the TRAMP C1 tumor actually decreased the number of Tregs in the spleen. Local irradiation of the TRAMP C1 tumors with 10 Gy increased the number of Treg in the spleen and the tumor (Kachikwu et al.).

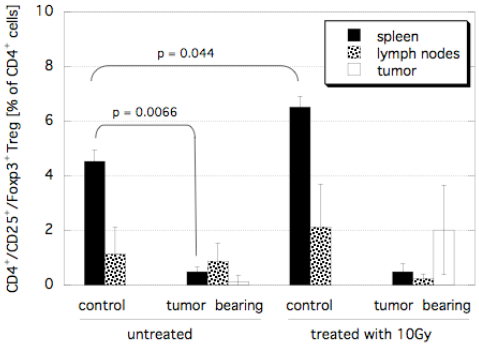

Fig. 3: The proportion of $CD4^+$ splenocytes that are Tregs decrease during prostate tumor progression. Detection of $CD4^+/CD25^+/Foxp3^+$ Treg cells in spleens, draining inguinal lymph nodes and subcutaneous TRAMP C1 tumors. Mice were either healthy (control) or tumor-bearing. The right leg, tumor bearing or not, was irradiated with 10Gy or left untreated. Cells were stained with antibodies for CD4, CD25 and Foxp3 and analyzed by flow cytometry. Data are mean of n=3 ± s.d. of 3 independent experiments.

The data suggest that Treg in vivo might be players involved in radiation-induced immunosuppression. We are currently performing experiments to determine if they are generated following immunization with irradiated DC, which would tie our observations of PSA presentation to this in vivo model.

We proposed as part of this investigation to determine if irradiation affects the expression of molecules involved in antigen presentation, not only by DCs but also by human prostate tumor cells. We have examined the former in depth previously but also examined the effect of radiation on human prostate cancer cell line, LNCAP, as proposed. These studies are linked to the effects of IL-3 and/or GM-CSF since we have shown that these can alter expression of co-stimulatory and adhesion molecules and result in superior antigen presentation. CD80, CD86, CD44, C-CAM and E-cadherin expression by LNCAP cells did not change significantly after irradiation. Addition of IL-3 (2 ng/ml for

1 week) or GM-CSF (0.45 ng/ml for 1 week) did not change the expression of CD44, CD80, CD86, C-CAM and E-cadherin, and radiation did not change this. However, HLA-A2 expression was decreased on radiation-treated cells. Furthermore, this did not happen in cells treated with IL-3 but not GM-CSF (Fig. 4). The level of PSA secreted was also measured following each treatment. As seen in Fig. 5, radiation treatment did not significantly change PSA levels in control or IL-3 treated cells, although IL-3 tended to decrease it. We have insufficient information to explain the change in HLA-A2 expression we observed but this could be highly significant in terms of tumor escape from immune surveillance. The effect of IL-3 is also of great interest and is consistent with what we observed years ago in animal tumors. Going forward we will be examining the effects of radiation on lipid rafts in DCs and prostate tumor cells and using adenoviral vectors to deliver IL-3 since we are convinced that this is a superior strategy.

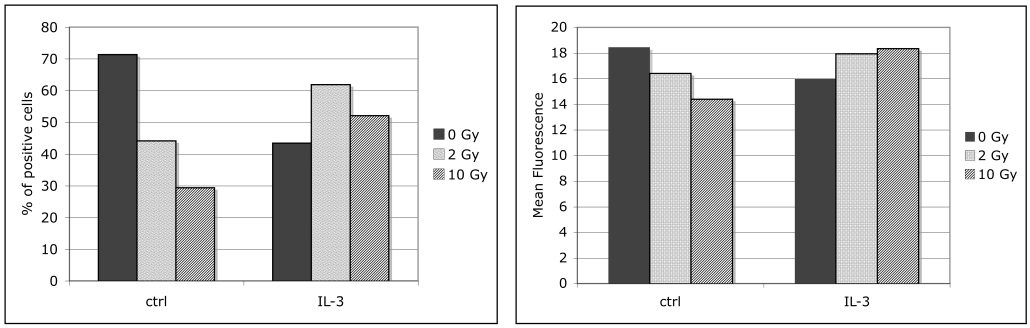

Fig. 4: Radiation effects on MHC class I (HLA-A2) expression on LNCAP. LNCAP cells were cultured with 2 ng/ml human IL-3 for 1 week, irradiated with 0, 2 and 10 Gy. HLA-A2 levels were assessed by flow cytometric analysis 48 hr after treatments. Data are shown as (a) percentage of HLA-A2 positive cells and (b) mean fluorescence.

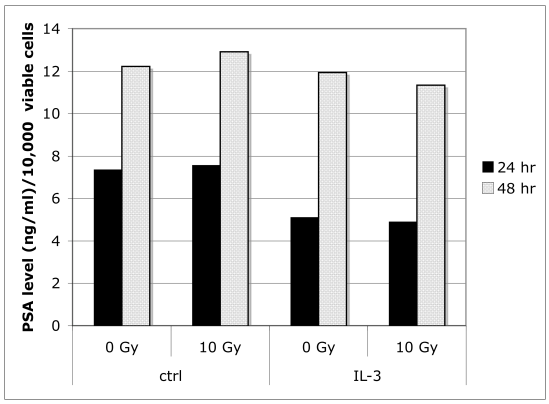

Fig. 5: PSA levels in LNCAP supernatants measured by ELISA 24 and 48hr after irradiation. Viable cells were counted using ATPLite assay in a separated setting. PSA level was normalized with viable cells

In addition, we have examined the impact of IL-3 on DC function. We have developed a treatment regimen with AdV-IL-3 combined with radiation therapy to examine this, as well as directly treating DCs (Fig.5, in 2006 annual report).

We have also developed a backup model using survivin as a prostate tumor-associated antigen because of the risk of focusing on one tumor antigen. We collaborated with a lab in Europe to monitor survivin-specific T cells after radiotherapy for prostate cancer. Circulating survivin-specific T cells increased as measured by tetramer analysis

suggesting that radiation can enhance prostate tumor antigen presentation (Fig.6; Schaue et al.).

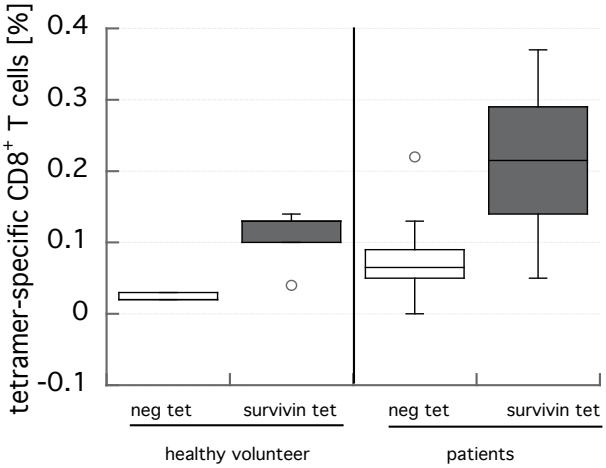

Fig. 6: Specificity of the survivin tetramer. Human samples stain relative higher with the tetramer for survivin when compared to staining with the irrelevant, negative tetramer ($p<0.005$). Data are percent $CD8^+$ T cells staining positive with the tetramer for survivin or the negative tetramer, respectively.

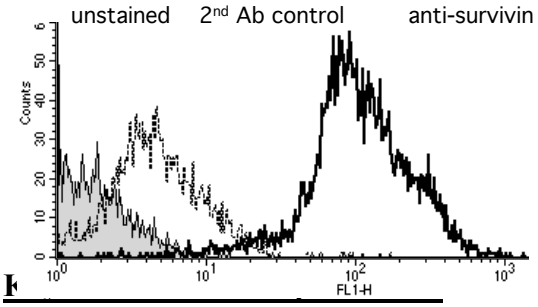

Fig. 7: Survivin expression in TRAMP C cells in vitro. A) Intracellular staining of fixed and permeabilized TRAMP C2/2.1 cells (1st antibody: rabbit anti human/mouse survivin; Cell Signalling, 2nd antibody FITC-goat anti rabbit, BD).

Our plan is to use humanized HLA-A2.1/Kb expressing C57BL/6 mice to look at the efficacy of DC-based PSA- and survivin-specific T cells responses against humanized tumors expressing human tumor-associated antigen. To do this we expressed the HLA-A2.1 molecule in TRAMP C1 murine prostate tumors using lentiviral vectors since we were unsuccessful with plasmid transfections (Fig. 7). This system has worked well, but remarkably the tumors do not grow in HLA-A2.1/Kb mice. This is at odds with our previous experience with 2 other tumor systems. We have performed these experiments multiple times using cloned and uncloned cell lines expressing differing levels of HLA-A2.1 and with TRAMP C2 tumors as well as TRAMP C1. These tumors do however grow in immune deficient mice, indicating that clonogenicity is not lost but rather they are immunogenic. This is extremely interesting since it indicates that we may have discovered a human prostate tumor specific rejection antigen that is presented in the context of HLA-A2.1. These studies are ongoing.

Key Research Accomplishments:

1. Study of the effects of IL-3 combined with radiation on DC function- completed.
2. Development of a survivin system- completed.

3. Study of the effects of radiation on endogenous processing of PSA by DC-completed.
4. Study of the effects of radiation on PSA peptide presentation by DC-completed.
5. Study of the effects of radiation and IL-3 and/or GM-CSF on PSA by prostate tumor cells- completed.

Reportable Outcomes – Manuscripts and Abstracts

Schaue D., Begonya Comin-Anduix, Antoni Ribas, Li Zhang, Lee Goodglick, James W. Sayre, Annelies Debucquoy, Karin Haustermans, and William H. McBride. T Cell Responses To Survivin In Cancer Patients Undergoing Radiation Therapy, submitted.

Kachikwu E.L., D. Schaue, Y. Liao, K. S. Iwamoto, J. S. Economou, and W. H. McBride. The Role of Naturally-Occurring T Regulatory Cells in Radiation-Induced Immune Modulation in Murine Prostate Cancer. Submitted.

Liao, Y., C-C Wang, L.H. Butterfield, J.S. Economou, A. Ribas, W.S. Meng, K. S. Iwamoto and W.H. McBride: Local irradiation of murine melanoma affects the development of tumor-specific immunity. Manuscript in preparation.

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Pajonk, F. and W.H. McBride: The proteasome in cancer biology and therapy. In: Cancer Drug Discovery and Development: Proteasome Inhibitors in Cancer Therapy, J. Adams, ed. The Humana Press, Inc., New Jersey, 2004, pp. 111-121.

Chiang, C-S., C.L. Hsieh, W.C. Liu, Y.T. Oh, W.H. McBride and J.H. Hong: Combing IL-3 gene immunotherapy with radiotherapy for prostate cancer. In: Abstracts of Papers for the 95th Annual Meeting of the American Association for Cancer Research, Orlando, Florida, 2004.

Pervan, M. and W.H. McBride: Mechanisms of proteasome inhibition by ionizing radiation. In: Abstracts of Papers for the 95th Annual Meeting of the American Association for Cancer Research, Orlando, Florida, 2004.

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Pervan, M. and W.H. McBride: Radiation targets within the proteasome. In: Abstracts of Papers for the 95th Annual Meeting of the American Association for Cancer Research, Orlando, Florida, 2004.

Chiang, C-S., C-H. Tsai, W-C. Liu, K-F. Hsieh, F-H. Chen, W.H. McBride and J-H. Hong: Enhancement of radiotherapy for murine prostate cancer by regulation of interleukin-3 gene expression using an adenovirus tetracycline turn-on system. In: Abstracts of Papers for the 96th Annual Meeting of the American Association for Cancer Research, Anaheim, California, 2005.

Reportable Outcomes – Presentations

1/07
McBride, W. H., Invited speaker: "Radiation affects the rate of protein degradation." University of Texas, Division of Radiation Biology/Department of Radiation Oncology Seminar Series.

5/07
McBride, W. H., "The proteasome in radiation responseS." 10th International Wolfsberg Meeting, Ermatingen, Switzerland.

7/7-7/12/07
McBride, W. H., Symposium speaker and abstract presentation: "Radiation affects on the composition and function of immune cells within the tumor microenvironment." 13th International Congress of Radiation Research, San Francisco, CA.

11/06
Liao, Y., Minisymposium speaker: "A Novel Mechanism of Radiation-Induced Immune Suppression." The 53rd Annual Meeting of the Radiation Research Society, Philadelphia, PA.

11/06
McBride, William H., Symposium speaker: "Does Radiation Signal "Danger" and Activate Tumor Immunity." The 53rd Annual Meeting of the Radiation Research Society, Philadelphia, PA.

4/06
McBride, William H. Invited Lecture: "Sense of Danger from Radiation," Department of Radiation Oncology, New York University, NY.

8/06
McBride, William H. Invited Speaker: "A Sense of Danger from Ionizing Radiation," International Workshop on Non-Targeted and Non-Linear Effects of Ionizing Radiation, Edinburgh, Scotland.

9/06
McBride, William H. Invited Lecture: "Effects of Radiation on the Immune System," Department of Radiation Oncology, New York University, NY.

4/27/05
Department of Radiation Oncology
New York University School of Medicine, New York, NY
Invited Lecture: "Sense of Danger from Radiation"

11/16/05
McBride, W.H., Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, Invited speaker: "The Proteasome in Cancer Biology and Therapy"

2/4/04
McBride, W.H., University of California, Riverside
Environmental Toxicology Program, Riverside, CA
Invited Seminar: "The Proteasome as a Sensor of Stress"

3/17/04
McBride, W.H., UCLA Department of Dentistry
Monthly Seminar, Los Angeles, CA
Invited Speaker: "The Proteasome as a Target for Cancer Therapy"

6/27-6/30/04
McBride, W.H., American Statistical Association Conference on Radiation and Health
Radiation in Realistic Environments: Interactions between Radiation and Other Risk Modifiers, Beaver Creek, CO
Invited Speaker: "The Proteasome and Radiation"
Session Discussant: "Interactions of Radiation with Genetic Factors"

9/20-9/22/04
McBride, W.H., Pharmacology & Therapeutics Department
Roswell Park Cancer Institute, Buffalo, NY
Invited speaker: "Radiation Effects Antigen Presentation to the Immune System"

10/23-10/28/04
McBride, W.H., 23rd Annual ESTRO Meeting, Amsterdam, The Netherlands
Abstract presentation: "The Proteasome as a Redox-Sensitive Target for Radiation Effects"

Personnel:
Vaughan Greer, Lab Helper
Kei Iwamoto, Co-PI
Yu-Pei Liao, Postgraduate Researcher
Kwanghee Kim, Staff Research Assistant
Frank Pajonk, Collaborator
Tyson McDonald, Graduate Student Researcher

Conclusions:

Ionizing radiation increased the ability of DC to present PSA peptide to T cells but decreased their ability to process PSA endogenously for presentation. Irradiation therefore modulates the way prostate tumor-specific antigen is presented to the immune system. Indeed, irradiated DC might serve as a tolerogenic signal to the immune system to block T cell responses. These are novel findings and we are the only ones performing studies of this nature. We have studied the ability of IL-3 on DC in a mouse model in the hope of reversing this form of radiation-induced immunosuppression of tumor antigen processing. Although the results with DCs were not too impressive, based on many other experiments we have performed, we believe that utilizing IL-3 as a gene therapy in combination with radiation therapy will still prove to be an effective strategy and that the effects on DCs will prove to be the key for this combination to work. Interestingly,

radiation effects of MHC class I expression by LNCAP human tumor cells could be prevented by IL-3 and this is another avenue to be explored. We have set up a new system using survivin as a tumor antigen. This will allow us simultaneously to explore antigen presentation to 2 distinct antigens within the one system. We have developed humanized mouse tumor models using genetic manipulation that surprisingly gave unexpected results. While they did not prove useful for the studies, they may inform on a human tumor-associated rejection antigen. These studies will give insights into which tumor antigens will prove superior for clinical translation. We have exerted a lot of effort in studying the radiation effects on immune responses in different aspects; some have open new avenues for further research. Although the grant period is ended, we will continue studies in our prostate cancer models and hope to find better ways to modulate radiation-induced immune responses and translate this into greater therapeutic effects for prostate cancer patients.

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Liao, Y., C-C Wang, L.H. Butterfield, J.S. Economou, A. Ribas, W.S. Meng, K. S. Iwamoto and W.H. McBride: Local irradiation of murine melanoma affects the development of tumor-specific immunity. Manuscript in preparation.

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Appendices: 2 submitted manuscripts follow.

Submitted for publication 2/2008 to the *Int. Journal of Radiation Oncology, Biology and Physics*

The Role of Naturally-Occurring T Regulatory Cells in Radiation-Induced Immune Modulation in Murine Prostate Cancer

Evelyn L Kachikwu MD[1*], Dörthe Schaue PhD[1*], Yu-Pei Liao PhD[1], Keisuke S. Iwamoto PhD[1], James S. Economou MD PhD[2], and William H. McBride DSc.[1]

[1]Radiation Oncology, [2]Surgical Oncology, David Geffen School of Medicine at UCLA, Los Angeles, USA.

* Authors contributed equally

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RUNNING HEAD: T regulatory cells and radiation

CONFLICT OF INTEREST NOTIFICATION: There are no actual or potential conflicts of interest.

ABSTRACT

PURPOSE: The control of locally invasive or metastatic disease remains the biggest challenge in the treatment of prostate cancer patients who receive standard radiation therapy and surgery. This low tumor burden group of patients could benefit most from the generation of specific anti-tumor immunity. One potential source of tumor-associated antigens is the extensive tumor cell death that accompanies radiation treatment. Unfortunately, this does not seem to generate strong anti-tumor responses, possibly due to an array of immune-suppressive mechanisms whose major purpose is to control autoimmunity.

METHODS: The $CD4^+CD25^+Foxp3^+$ subset of immunosuppressive T regulatory cells (Treg) have long been suspected as aiding immune evasion by cancer, although little is known as to the effects of radiation therapy on these cells. Here, we followed their fate after localized radiation treatment of Transgenic Adenocarcinoma of the Mouse Prostate C1 (TRAMP C1).

RESULTS: $CD4^+CD25^+Foxp3^+$ Treg cells were detected in various immune organs and in TRAMP C1 tumors growing in a s.c. site. Interestingly, the proportion of $CD4^+$ Treg cells in the spleen declined dramatically in the presence of a s.c. tumor. Local tumor irradiation increased the proportion of $CD4^+$ Tregs in the spleen and these cells exhibited a stronger suppressive phenotype once isolated. Systemic elimination of Tregs greatly enhanced tumor regression following local tumor irradiation, which could be clinically important.

CONCLUSIONS: We conclude that local radiation treatment of tumors has systemic effects on Treg proliferation and activity and/or Treg trafficking and

might exacerbate tumor immune evasion. Targeting this population may allow radiotherapeutic enhancement through immune modulation.

KEYWORDS: regulatory T cells; radiation therapy; transgenic adenocarcinoma of the mouse prostate; anti-CD25 therapy

1 INTRODUCTION

Radiation Therapy (RT) along with surgical de-bulking of larger tumors is the first line treatment option for prostate cancer patients. It results in a five-year disease-free survival approaching 95%. However, locally invasive or metastatic disease is inherently more difficult to control and demands systemic approaches. Immunotherapy could be an attractive additional treatment for these patients.

Our experience is that radiation-induced cell death alone generates a limited amount of anti-tumor immunity [1, 2], which is unlikely to translate into a clinical response. Immunotherapies in combination with standard treatments therefore appear to be the way forward. For RT and immunotherapy to be integrated into a protocol it is imperative to understand how radiation affects the immune system beyond killing lymphocytes. There is increasing evidence that radiation has a spectrum of immune-modulating potential ranging from enhancing immunity to favoring tolerance. For example, we have shown that radiation inhibits dendritic cell processing of antigen through the endogenous pathway but enhances exogenous processing [3]. Also, the immunosuppressive M2 form of tumor-associated macrophages (TAM), which is normally dominant in tumors [4, 5], is enhanced post-irradiation, suggesting that RT does not generally switch an immunosuppressive tumor microenvironment to one that is more immunostimulatory [2, 6, 7].

Of the various immune escape mechanisms that have been proposed, T regulatory cells (Treg) have long been suspected as being major players [8, 9]. Many tumors are heavily infiltrated with Treg cells [10-14]. These cells are crucial

for the maintenance of peripheral tolerance and they execute their suppressive effect on other T cells through cell-to-cell contact mechanisms, often aided by cytokines such as IL-10, IL-4 and TGF-β [15-21].

There are different types of Tregs. Some are inducible (adaptive) by low-dose antigen or TGF-β, such as the $Foxp3^-$ Tr1 Treg cells and the Th3 cells that secrete IL-10 and TGF-β [22-24]. However, the most important Tregs currently known are naturally occurring $CD4^+CD25^+Foxp3^+$ cells (nTregs) that exit the thymus as a functionally mature T cell population already primed for suppressive function [25]. The forkhead box transcription factor (Foxp3) acts as a master switch in the regulation and development of $CD4^+$ Tregs [26]. Both Foxp3-transduced T cells and $CD4^+CD25^+$ Tregs have marked suppressor activity [27, 28].

There are many unanswered questions concerning the effect of cancer therapy on Tregs, including their relationship to induced suppressor T cells that were shown in older studies to be more radiosensitive and chemosensitive than other T cell subsets [29, 30]. The development of more specific markers for Tregs, namely Foxp3 and CD25 allowed us to revisit the concept of radiation-induced alteration of this suppressor T cell subpopulation within the context of a tumor model. We investigated the effect of RT on intra-tumoral and systemic nTreg cells in C57Bl/6 mice bearing the Transgenic Adenocarcinoma of the Mouse Prostate C1 (TRAMP C1).

2 METHODS and MATERIALS

2.1 Mice and cell lines

Female 6-8 week old C57Bl/6, and C57Bl/6 mice engineered with a bicistronic Foxp3 allele linking the expression of Foxp3 with that of the green fluorescent reporter protein EGFP ($Foxp3^{EGFP}$ mice) were a kind gift from Dr. Chatila, UCLA, and were bred and maintained in a defined-flora, pathogen-free environment in the American Association of Laboratory Animal Care (AALAC)-accredited Animal Facility of the Department of Radiation Oncology, University of California at Los Angeles. Experiments adhered to all local and national animal care guidelines. TRAMP C1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and initially cultured in DMEM (Mediatech, Herndon, VA) with 10% FBS (Omega Scientific, Tarzana, CA), 10,000IU penicillin, 10,000µg/ml streptomycin, 25µg/ml amphotericin (Mediatech), 5µg/ml insulin and 10^{-8}M dihydrotestosterone (Sigma-Aldrich, St.Louis, MO). TRAMP C1 cells were routinely maintained in vivo as subcutaneous tumors. TRAMP C1 tumors were harvested and digested with collagenase D (Roche) and DNAse 1 (Type IV; Sigma), and clumps removed by filtration. Mice were inoculated s.c. on the outer right thigh with $1x10^5$ freshly harvested TRAMP C1 tumor cells. Tumors reached 4-7mm average diameter within 2-3 weeks. The dendritic cell line DC2.4 (a kind gift from K.L. Rock) was maintained in RPMI 1640 medium (Mediatech), with 10% FBS (Omega Scientific), 1% antibiotics-antimycotic solution (Mediatech) and 50µM 2-Mercaptoethanol (Sigma).

2.2 Radiation treatment

Mice were anaesthetized with an i.p. injection of Nembutal, and positioned in a Lucite jig with lead shielding the body, except for the right leg, which was irradiated using a Gamma cell 40 irradiator (Cs-137 source; Atomic Energy of Canada Ltd., Ottawa, Canada) at a dose rate of approximately 67cGy/min. Sham irradiation or 10Gy was given to the right thigh (tumor-bearing or control), 4 mice per group. Mice were allowed to recover and were sacrificed 48h later for all experiments involving Treg enumeration (2.3) and Treg isolation (2.4) or tumor growth followed with time, as appropriate.

2.3 Treg enumeration

Lymphocytes were harvested from spleens and draining inguinal lymph nodes, depleted of red blood cells by ACK treatment (0.83% (w/v) NH_4Cl, 0.14% (w/v) $KHCO_3$, 0.002% (w/v) Na_2EDTA, PH 7.3), and re-suspended as a single cell suspension. Tumor cells were prepared as above. Lymphocytes were stained for Treg markers Foxp3, CD4 and CD25 according to the manufacturer's recommendation with minor modifications (eBioscience). Briefly, $2x10^6$ cells were fixed in Fix/Perm working solution, permeabilization buffer added and non-specific binding sites blocked with 1% (w/v) BSA in PBS prior to staining with FITC-Foxp3 (clone FJK-16s, eBioscience), PE-CD25 (clone PC61, BD Pharmingen), and PE-Cy5-CD4 (clone L3T4, BD Biosciences Pharmingen). Flow

cytometric analysis was performed on 100,000 $CD4^+$ events using a FACS Calibur system (BD Biosciences, Mountain View, CA).

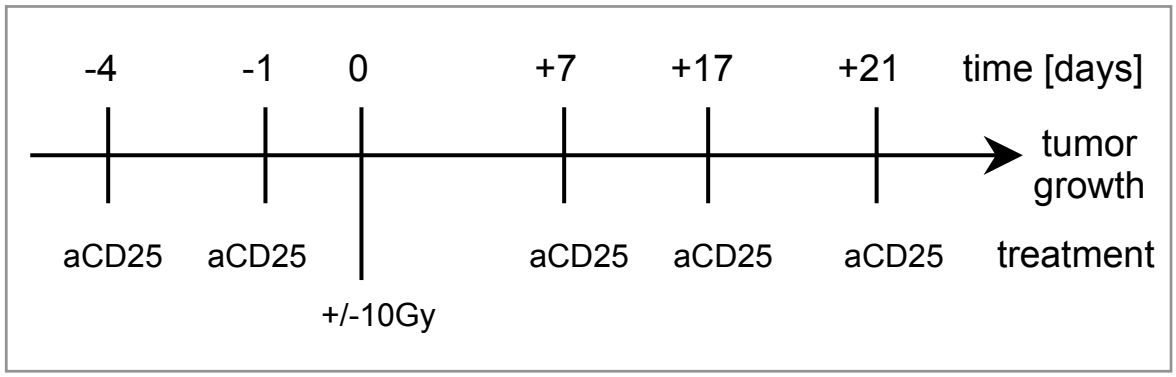
2.4 Proliferation assay

Tregs were isolated from spleens and tumors of tumor-bearing mice and from spleens of control mice 48h post treatment. Magnetic sorting on an autoMACS separator was used to isolate $CD4^+CD25^+$ Tregs (Miltenyi Biotec, Auburn, CA). $CD4^+$ cells were obtained by negative selection, followed by positive selection to obtain the $CD4^+CD25^+$ fraction. Aliquots of the two fractions were stained for CD4 and CD25 as above and analyzed by flow cytometry to assess purity prior to the proliferation assay. Tregs from the different treatment groups were co-cultured with DC2.4 cells at 10^4:10^3 in 96-well round-bottom plates in triplicates for three days. Control groups included Tregs and DC2.4 alone. Wells were pulsed with 1μCi of ^{3}H-Thymidine for the last 18 hours. Cells were harvested onto glass microfibre filters and beta-emission counted in a Wallace 1450 Microbeta Trilux Counter.

2.5 $CD25^+$ cell depletion and tumor protection

Two groups of mice, half with small tumors (<300mm^3 volume), and the other with large tumors (>350mm^3), were randomized to receive 200μl of anti-CD25 mAb i.p. (PC61, BioExpress, NH) or PBS on days -4, -1, +7, +17, and +21, and to receive +/- 10Gy irradiation to the tumor site on day 0. All groups contained 2-4 mice. Tumors were measured prior to starting treatment, and every

three to four days thereafter, using vernier calipers. Tumor volumes were calculated using the formula: width^2 x length x 0.52.



2.6 Statistical analysis

Data were analyzed for statistical significance with the paired Student's t-test. Significance was assessed at the 5% level.

3 RESULTS

3.1 Treg profiling

Flow cytometric analysis of splenocytes from untreated C57Bl/6 mice typically identified 20% as being $CD4^+$. Of these about 15% were $CD25^+$. This was similar to the 17% that stained positive for intracellular Foxp3, i.e. 3-4% of total splenocytes. Triple staining identified 11% of $CD4^+$ T lymphocytes as co-expressing the Treg markers CD25 and Foxp3 (Figure 1); i.e. ~80% of $CD4^+CD25^+$ T cells were $Foxp3^+$. About one third of the triple-staining $CD4^+CD25^+Foxp3^+$ cells (or 4% of all $CD4^+$ cells) were activated, as judged by high levels of expression of the IL-2 receptor CD25.

We compared these figures with those obtained using $Foxp3^{EGFP}$ C57Bl/6 mice in which the functionally activated Foxp3 promoter drives EGFP expression. As for Foxp3 staining, 5% of $Foxp3^{EGFP}$ splenocytes expressed EGFP and they belonged almost exclusively (97%) to the CD4 lineage (Figure 2A and B). About 11% of $CD4^+$ splenocytes were $CD4^+/CD25^+/Foxp3^+$ (Figure 2C and D) although, only about half of the $Foxp3^+$ cells were $CD25^+$. Most of the $Foxp3^+CD25^+$ cells were $CD25^{high}$ (Figure 2B), suggesting that they were activated, but Foxp3 clearly can be active in $CD25^-CD4^+$ cells, which has been reported in the literature [25]. Overall, however, there was a high degree of overlap between the $CD4^+/CD25^+$ and the $CD4^+/Foxp3^+$ population and we conclude that typically about 10% of $CD4^+$ splenocytes in C57Bl/6 mice express Treg markers and that our intracellular Foxp3 staining was accurately reflecting Foxp3 function.

3.2 Tregs in tumor-bearing mice

The effect of a syngeneic TRAMP C1 tumor growing in a s.c. site on the proportion of $CD4^+CD25^+Foxp3^+$ Tregs in the spleens of C57Bl/6 mice was assessed, as was the effect of local tumor/leg irradiation with 10Gy. In the experiment shown in Fig 3, the number of Tregs was slightly lower than usual in control spleens, but was dramatically decreased (to <1% of $CD4^+$; p=0.0066) in mice bearing TRAMP C1 tumors of about 10mm diameter. This occurred in the absence of noticeable splenomegaly that can develop in mice with large tumors. Local irradiation delivered to the tumor had little effect on the low levels of splenic Tregs as assessed 48hrs later (Figure 3). These findings were reproduced in multiple experiments and in $Foxp3^+$ transgenic mice bearing TRAMP C1 tumor (data not shown). Interesting, irradiation of the right leg alone in the absence of tumor significantly, but not dramatically, increased the number of splenocytes that stained for Treg markers (p=0.044; Figure 3).

The low level of splenic Tregs in mice bearing TRAMP C1 tumors was not seen in draining lymph nodes, which in healthy and tumor-bearing animals had comparably low fractions of about 1% of $CD4^+$ cells that stained $CD25^+Foxp3^+$ (p=0.8). If anything, irradiation of the leg increased the proportion of Tregs in the inguinal lymph node, which were in the radiation field, and decreased it in tumor-draining lymph nodes, but these changes were not statistically significantly.

Intratumoral $CD25^+Foxp3^+$ cells as a percent of $CD4^+$ lymphocytes was very low in control tumors (Figure 3) and increased slightly, but variably, forty-eight hours after tumor irradiation with 10Gy.

3.3 $CD4^+CD25^+$ Tregs exhibit suppressive activity in vitro

To test $CD4^+CD25^+$ Tregs from spleens and tumors of control and/or tumor-bearing mice for functional activity, they were isolated by $CD4^+$ enrichment with subsequent magnetic bead sorting for $CD25^+$ cells. The initial negative selection essentially eliminated $CD4^-$ cells from the sample (Figure 4A) while $CD4^{low}$ and $CD4^{high}$ staining cells remained (Figure 4B,C). Positive selection for CD25, yielded a population that were 84% $CD4^+CD25^+$ and largely (74%) $CD4^{high}CD25^+$ (Figure 4C). The number of $CD25^+$ cells in the $CD4^+CD25^-$ fraction was negligible (Figure 4B).

We assessed the ability of isolated $CD4^+CD25^+$ T cells to suppress proliferation of the DC2.4 dendritic cell line in vitro. T cell proliferation was negligible but DC2.4 proliferation was inhibited by their presence to the same extent whether they came from control spleens, spleens from tumor-bearing mice, or from within the tumor (Figure 5). Irradiation of either the leg or the tumor increased the suppressive action of the purified Treg populations over the sham irradiated controls (Figure 5), but none of the differences reached individual statistical significance.

3.4 $CD4^+CD25^+$ Treg cells mediate tumor evasion in vivo

Anti-CD25 monoclonal antibody was used to deplete Tregs to determine if they contributed to suppressing anti-tumor immunity within a radiation therapy setting. Antibody treatment alone caused, at best, transient tumor growth delay

and RT alone was also largely ineffective (Figure 6A). However, mice receiving antibody injections in combination with RT had the longest tumor growth delay and showed signs of transient regression ($p=0.003$; day7; Figure 6A). By the time all animals in other groups had to be euthanized because of large tumors, 60% of those in the combined therapy group had acceptable tumor volumes (<1.5cms diameter)(Figure 6B).

4 DISCUSSION

The frequency of $CD4^+/CD25^+/Foxp3^+$ Treg cells that we detected in spleens of C57Bl/6 mice, either by triple staining or by double staining using Foxp3 transgenic mice, was about 10% of $CD4^+$, which is well within the previously published range, indirectly validating our protocol [25, 31]. The marked decline in splenic Tregs in mice growing TRAMP C1 tumors in a distant s.c. site was somewhat surprising, but highly reproducible. This decrease was accentuated as tumor growth progressed (not shown) and this may ultimately relate to changes in the cellular composition particularly the decrease in $CD4^+$ T cells sometimes seen during tumor progression [32]. Recently we observed similar low proportions of circulating Treg cells (as % of $CD4^+$ T cells) in the peripheral blood of patients with prostate and colorectal cancer. Since Treg cells are equally distributed between spleen and secondary lymphoid organs [33], we can make the assumption that what we observed in the murine spleen and the human peripheral blood is a consequence of tumor-bearing and may not be an uncommon phenomenon, in spite of the fact that the literature is dominated by reports on increased levels of peripheral Treg cells in many cancer patients. What is clear is that tumors have systemic effects that can influence the Treg pool. Whether this effect varies depending on the tumor type and the factors they release, will require further investigation. It may be that in some situations Treg cells are depleted or re-locate to other sites as a result of tumor-derived factors. We have no evidence to support such a scenario but Treg recruitment to tumors has been observed in mice [34] and in patients [12]. Curiel and colleagues also

reported a tendency of Tregs to avoid the draining lymph nodes, an effect that escalated with disease progression.

Local tumor irradiation, if anything, increased the frequency of Tregs in the tumor, but had little effect on the depressed levels in the spleen or draining lymph node. T cell frequencies alone can be vastly misleading in terms of efficacy and outcome and Radoja et al. [32] recently demonstrated that T cell frequencies and T cell function on a single cell basis do not necessarily correlate. Our finding that the functional activity of Tregs in the spleen and tumor of these animals also seemed to be enhanced is therefore important. Traditionally, the possibility of modulation of immune cell function by ionizing radiation has been somewhat overshadowed by the focus on its cytotoxic action. However, it is able to induce and activate the powerful immune-suppressive cytokine TGF-β [35], which is known to enhance Treg actions [36] and the concept that Tregs may be activated in response to radiation damage must be considered plausible.

The possible recruitment of Tregs to the tumor following local irradiation is something that requires further evaluation within the context of other radiation-induced changes within the tumor immune microenvironment [37]. Increased expression of adhesion molecules have been described following tissue irradiation [38, 39], as have waves of inflammatory cytokine expression [40, 41]. These could impact the involvement of Tregs and other immune cells within the immunological synapse. The extent to which this happens following tumor irradiation is still controversial, but the concept of irradiation inducing danger signals to modify DC function is intriguing. The recent observation by Apetoh [42]

that innate immune responses can be activated by RT that in turn facilitate adaptive anti-tumor immunity therefore should not be surprising and make a strong case for RT to be thought of as an immunoadjuvant [43], although how powerful and how universal this phenomenon is has yet to be established. Our observation that local leg irradiation increased Tregs in the spleen in control mice but did not affect the depressed levels in tumor-bearing mice, suggest that local microenvironmental factors may outweigh the influence of radiation-induced effects and that immune therapies aimed at altering the tumor-host relationship within the irradiated site will become critical if RT is to be used in conjunction with cancer immunotherapy. Our data that mice receiving combined RT and anti-CD25 antibody, showed substantial tumor regression that exceeded either treatment alone, is most encouraging. This has been demonstrated in other tumor models [44] and it opens exiting new avenues for future cancer therapies [43, 44] that will be enhanced if they are used to unmask the potential of the RT to boost systemic immunity to help achieve local and distant tumor control.

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Figure Legend

Figure 1: Characteristic FACS profile of splenocytes stained for Treg markers CD4 (FL3), CD25 (FL2) and Foxp3 (FL1). **A)** Forward-side scatter of 100,000 scanned events including debris (D), B cells (B), T cells (T), macrophages (M) and granulocytes (G). **B)** 21% of splenocytes are CD4 positive (gate R1, high FL3) and these are largely lymphoctyes (forward scatter 200-400). **C)** 11.4% of $CD4^+$ cells are $CD4^+/CD25^+/Foxp3^+$ Tregs (upper right quadrant).

Figure 2: Treg phenotyping in splenocytes of $Foxp3^{EGFP}$ mice. Splenocytes were stained for CD4 (FL3) and CD25 (FL2) and assessed by flow cytometry. **A)** dot blot of Foxp3 (FL-1) vs forward scatter. 5% of splenocytes expressed Foxp3 (gate 1). **B)** CD4 vs CD25 dot blot of $Foxp3^+$ cells. The majority of $Foxp3^+$ cells (gate 1) were also positive for CD4 (upper left and upper right quadrant). **C)** FL-3 histogram of splenocytes. Almost 20% of splenocytes are $CD4^+$. **D)** 11% of $CD4^+$ cells are $CD4^+/CD25^+/Foxp3^+$ Tregs (upper right quadrant).

Figure 3: The proportion of $CD4^+$ splenocytes that are Tregs decrease during tumor progression. Detection of $CD4^+/CD25^+/Foxp3^+$ Treg cells in spleens, draining inguinal lymph nodes and subcutaneous TRAMP C1 tumors. Mice were either healthy (control) or tumor-bearing. The right leg, tumor bearing or not, was irradiated with 10Gy 48h prior to analysis or left untreated. Cells were stained with antibodies for CD4, CD25 and Foxp3 and analyzed by FACScan. Data are mean of n=3 ± s.d. of 3 independent experiments.

Figure 4: Isolation of $CD4^+/CD25^+$ Tregs from C57Bl/6 spleen. Splenocytes were sorted with the MACS (miltenyi biotec) system of $CD4^+$ T cell pre-enrichment (negative selection) and positive CD25 (PE) selection. Cellular fractions were then stained for CD4 (FL3). Data are FL3 vsFL2 dot blots of **A)** whole spleens, **B)** $CD4^+/CD25^-$ fraction and **C)** $CD4^+/CD25^+$ fraction.

Figure 5: $CD4^+/CD25^+$ Tregs suppressed cell proliferation in vitro. Tregs were isolated from spleens and tumors of irradiated or non-irradiated mice 48h post treatment. Splenic Tregs from healthy mice served as control. Tregs were mixed with the responders DC2.4 and proliferation was assessed according to ^3H-Thymidine incorporation. Data are % inhibition of DC2.4 proliferation ± s.d. of n=3.

Figure 6: Treatment of TRAMPC1 tumors with anti-CD25 antibody (aCD25 Ab) in combination with 10Gy (RT) local irradiation caused tumor growth delay. Tumor-bearing mice were given aCD25 Ab or PBS i.p. on day –4, -1, +7 and +14. Tumors were irradiated on day 0 with 10Gy or left untreated. **A)** Tumor size as mean mm diameter (2 dimension) of n = 2-6 ± s.d. **B)** Survival data with % of mice per treatment group alive.

T Cell Responses To Survivin In Cancer Patients Undergoing Radiation Therapy

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RUNNING TITLE: T Cell Responses To Survivin After Radiation

KEY WORDS: radiation therapy, survivin, colorectal cancer, prostate cancer, immunity

ABSTRACT

PURPOSE: The goal of this study was to determine if radiation therapy of human cancer enhances or diminishes tumor-specific T cell reactivity. This is important if immunotherapy is to be harnessed to improve the outcome of cancer radiotherapy.

EXPERIMENTAL DESIGN: Lymphocytes were isolated from colorectal cancer (CRC) patients, before, during, and after presurgical chemoradiotherapy (CRT). Similar samples were taken from prostate cancer patients receiving standard radiation therapy (RT). The level of $CD8^+$ T cells capable of binding tetramers for the tumor-associated antigen survivin, which is over-expressed in both cancer types, were enumerated in HLA-A*0201 patient samples. $CD4^+$, $CD25^{high}$, $Foxp3^+$ cells were also enumerated to evaluate therapy-induced changes in $T_{regulatory}$ cells. For CRC patients, most of whom were enrolled in a clinical trial, pathological response data were available, as well as biopsy and resection specimens, which were stained for cytoplasmic and intranuclear survivin.

RESULTS: Survivin-specific $CD8^+$ T lymphocytes were detected in the peripheral blood of CRC and prostate cancer patients and increased after therapy in some, but not all, patients. Increases were more common in CRC patients whose tumor was down-staged after CRT. Biopsy specimens from this cohort generally had higher nuclear:cytoplasmic survivin expression. $T_{regulatory}$ cells generally increased in the circulation following therapy, but only in CRC patients.

CONCLUSION: This study indicates that RT may increase the likelihood of some cancer patients responding to immunotherapy and lays a basis for future

investigations aimed at combining radiation and immunotherapy.

1 INTRODUCTION

Management of cancers of the rectum and the prostate relies heavily on radiation therapy (RT), but later stage disease is often hard to control. Harnessing the immune system to assist in the elimination of cancer cells within, and outside, the radiation field could be beneficial in such situations but this will require knowledge of the effects of RT on tumor-specific immunity in humans, about which little is known. Preclinical data are not very helpful, suggesting consequences ranging anywhere from favoring tolerance to enhancing immunity (1-6).

Here, we used a sensitive tetramer assay for the tumor-associated antigen survivin to ask what happens to tumor-specific T cell responses in colorectal and prostate cancer patients during and after RT. Survivin is highly expressed in many human cancers, but is largely undetectable in most normal tissues (7, 8). It augments cell proliferation and survival (9), either by inhibiting caspase 9 and hence apoptosis (10) or by directing chromosome movement during mitosis (11). Its location in the cytoplasm or nucleus may be crucial in determining its function as well its prognostic potential (12-14). Importantly, it has been shown to be associated with resistance to therapy, including RT (9, 15).

The evidence that survivin is immunogenic is strong. In preclinical models, survivin-reactive $CD8^+$ cytotoxic T lymphocytes (CTL) can be generated in vitro that efficiently lyse target cells and confer tumor protection on adoptive transfer in vivo (16-19). In humans, survivin-reactive T-cells can be detected in primary breast cancer and melanoma lesions, and in lymph nodes and blood of cancer

patients (20, 21), who also develop anti-survivin antibodies (22). Both animals and patients respond to vaccination with this antigen (23-25).

Unfortunately, clinical experience indicates that adaptive anti-tumor immune responses generally fail to translate into measurable tumor regression. This has been ascribed to a variety of immune escape mechanisms, one of which is the presence of $T_{regulatory}$ cells (26). Different types of $T_{regulatory}$ cells have been described (27-29) but the $CD4^{+}$, $CD25^{high}$, $Foxp3^{+}$ subset are generally considered important in suppressing anti-tumor immunity (30). Human tumors are frequently infiltrated with such cells (31-33), which suppress effector T cells through multiple mechanisms (34, 35). While older studies in mice showed that tumor-induced suppressor T cells were more radiosensitive than other T cell subsets (36), there is little data on the radiosensitivity of $T_{regulatory}$ subsets in humans.

This study asks whether survivin-specific cytotoxic $CD8^{+}$ T cells can be detected in patients with prostate or colorectal cancer, whether cancer treatment with RT or CRT alters the tumor-specific immune status in these patients, whether the level circulating $T_{regulatory}$ cells are affected, and whether any immune parameters correlate with pathological tumor regression.

2 Methods

2.1 Chemicals

The following were used: Ficoll-Paque (GE Healthcare Bio-Sciences; Uppsala, SE), human AB serum (OmegaSci.; Tarzana, CA), dimethyl sulfoxide and DNAse (Sigma; St. Louis, MO), RPMI-1640 medium with L-glutamine and antibiotics (Fisher; Pittsburg, PA), tetramers and anti-CD8 antibody (T8-FITC; clone SFCI21Thy2D3) (Beckman Coulter, Inc.; San Diego, CA), 7-Amino-Actinomycin D (7-AAD), FITC-anti-human HLA-A2 (clone BB7.2), PE-Cy5™-anti-human CD25 (clone M-A251) and R-PE-anti-human CD4 (clone RPA-T4) (Pharmingen; San Diego, CA), FITC-anti-human Foxp3 (clone hFOXY), PE-Cy5-anti-human Foxp3 (clone PCH101), FITC-anti-human CD4 (clone OKT4) and PE-anti-human CD25 (clone BC96) (eBiosciences; San Diego, CA), rabbit anti-human survivin (clone NB500-201) (Novus Biologicals; Littleton, CO), and biotinylated anti-rabbit (BA-1000) (Vector; Southgate, UK).

2.2 Patients & sample collection

Blood samples came from patients with colorectal (CRC; n=28) or prostate cancer (n=20) in the University Hospital Leuven, Gasthuisberg, Belgium, with local IRB approval and consent. Prostate patients received conventional RT. All but 3 with CRC were part of a phase II randomized, double-blind, placebo-controlled clinical trial with the COX-2 inhibitor Celecoxib described previously (37). Preoperative CRT was 45Gy in 25 fractions with continuous 5-FU infusion. Patients were randomized to Celecoxib (2x400mg/day) or placebo prior to

surgery, which was on week 6. Blood samples were taken into BD vacutainer® CPT™ tubes (BD, USA) before, during (week 3), and after CRT (week 5). Peripheral blood mononuclear cells (PBMCs) were isolated following gradient centrifugation and frozen in human AB serum containing 10% (v/v) DMSO. Frozen blood samples were shipped on dry ice and stored in liquid nitrogen upon arrival in the USA. Serial samples of individual patients were assayed for tetramer and $T_{regulatory}$ cell staining (see below) on the same day. PBMCs from 8 healthy volunteers were isolated on Ficoll-Paque™ at UCLA and stored as above. Pretreatment biopsies (21) and residual tumor surgery specimens (10) CRC patients were fixed tissue and processed for immunohistology. This trial was delayed because of COX-2 inhibitor safety issues, resulting in some incompleteness of data.

2.3 HLA-A2 testing

PBMCs from patients and healthy subjects were thawed by dilution in pre-warmed RPMI-1640 medium with 10% (v/v) human AB serum. Cells were treated with DNAse, washed and re-suspended at $5x10^6$ cells/ml in human AB serum. 20µl ($1x10^5$) were stained with 1µl of FITC anti-HLA-A2 antibody for 30 minutes at 4°C, washed and re-suspended in 300µl PBS for flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA).

2.4 Tetramer-binding assay

$1x10^6$ cells in human AB serum (200µl) from HLA-A*0201-positive subjects were stained with 8µl of the MHC tetramer for the HLA-A2-restricted survivin epitope Sur1M2 (LMLGEFLKL) (21) along with 8µl of anti-CD8 antibody. Sample volume permitting (28 samples), a MHC class I human negative tetramer with no known specificity that does not bind $CD8^+$ T cells of any HLA allele (Beckman Coulter) was used to determine background PE fluorescence. After incubation for 30 minutes at room temperature and washing, samples were re-suspended in PBS. 7-AAD was added to detect non-viable cells 5-10 minutes prior to flow cytometry. PBMCs from a single HLA-A*0201-positive volunteer were run as an internal control for each assay. $1\text{-}2x10^5$ events were accumulated. Quality control required ≥10,000 viable events and ≥2,000 $CD8^+$ T cells.

The gating strategy was:

1) plot FL3 , set viability gate (gate 1); (fixed cells as control)
2) plot FL1 vs. FSC of population in gate 1; set gate 2 for $CD8^{high}$ lymphocytes, excluding NK cells ($CD8^{low}$)
3) plot FL-2 vs. FL-1 of cells in gate 1 and 2 (viable $CD8^{high}$ lymphocytes). Samples of one healthy volunteer stained with negative tetramer were used to set an arbitrary FL-2 lower limit of 0.03% double positives (38).

Two batches of survivin tetramer were used and correction had to be made for differences in binding. Positivity was based on a HLA-A*0201^+ healthy volunteer

having 0.053% ±0.023 reactive $CD8^+$ T cells for one batch and 5 HLA-A*0201^+ healthy volunteers having 0.100% ± 0.075 for the other (Table 1). The low-limit for a positive value was taken to be the mean ± 2SD of these values, i.e. 0.099% for batch 1 and 0.25% for the second batch.

2.5 $T_{regulatory}$ cell staining

$CD4^+$, $CD25^{high}$ cells with intracellular $Foxp3^+$ were examined. For most samples, $1x10^6$cells were stained in 100µl human AB serum with 20µl FITC-anti-human CD4 and 20µl PE-anti-human CD25 and incubated for 30 minutes on ice. Cell were washed and incubated in 1ml of fixation/permeabilization buffer for 45mins on ice, washed twice and re-suspended in 2% v/v normal rat serum in 100µl of permeabilization buffer. 20µl of PE-Cy5-anti-Foxp3 (clone PCH101) was added followed by 30mins on ice. Cells were washed, re-suspended in 200µl of 10% FBS and analyzed by flow cytometry. In an earlier protocol, the antibody cocktail containing PE-$Cy5^{TM}$-anti-CD25, R-PE-anti-CD4 and the first generation FITC-anti Foxp3 antibody (clone hFOXY) were applied simultaneously after fixation & permeabilization. PBMCs from one volunteer served as an internal control for each assay. If possible, $1x10^5$ events were accumulated. Quality control required all acquired data to be ≥70% viability and ≥2,000 $CD4^+$ T cells.

The gating strategy was:

1) FL-1 vs. FSC of all events, set gate 1 for $CD4^+$ cells, excluding debris and monocytes ($CD4^{low}$).

2) FL2 vs. FL1 of population in gate 1, set gate 2 for $CD4^{+}CD25^{high}$ double positive lymphocytes.
3) Plot FL-3 of cells in gate 1 and 2 to determine fraction of $CD4^{+}CD25^{high}Foxp3^{+}$ triple positive cells.

2.6 Immunohistochemistry for survivin

Tissues were deparaffinized at 75˚C (30mins), xylene, and decreasing percentages of alcohol and washed 5 times in water. Sections were steamed in citrate buffer (100mM, pH 6.0; 25mins) and washed 5 times with PBS. Endogenous peroxidase was blocked with 3% H_2O_2 in methanol (15mins), washed, and incubated with 5% normal goat serum in 0.05% Tween 20 in PBS. Polyclonal rabbit anti-human survivin (1:200) was added and slides were incubated (30mins-room temp; ON at 4˚C). Biotinylated anti-rabbit Ig (1:200) in 5% normal goat serum in 0.05% Tween 20 was added (40mins) followed by DAB (3mins). After counterstaining with hematoxylin (10secs), slides were dehydrated and mounted. The identity of slides was blinded through a number code and scored in Belgium. Cytoplasmatic survivin was scored for percentage tumor tissue staining positive and for intensity on a scale from 0-3. For the nuclear staining we only scored the percentage, since the intensity was did not vary.

2.7 Statistics

All but one set of data were analyzed for statistical significance with the sign test and, after a square root transformation, with a Student's t-test (39).

Whether or not the level of circulating $T_{regulatory}$ cells was significantly different from control levels was determined with the Wilcoxon signed ranks test (39). Statistical significance was at the 5% level. In general, pooled patient data sets were compared as cohorts to the healthy control levels. Longitudinal responses for each patient compared outcome values (during or after) to individual baseline levels (before) and were then summarized for the whole cohort.

Box-plots are box-whisker diagrams summarizing the distribution of data as 1) the box spanning the 75-25% percentile and 2) the median (line), 3) the minimum and maximum (whiskers above and below the box) and 4) individual outlying data points (open circles).

3 Results

3.1 Survivin-specific $CD8^+$ T cells

Of 49 patients, 28 (57%) were HLA-A*0201 positive and eligible for tetramer analysis. However, 2 samples did not meet the quality control standards and were excluded from analyses.

Levels of survivin tetramer-reactive $CD8^+$ cells were significantly higher in patients than 5 healthy controls (Supplementary Figure, $p<0.001$), indicating the presence of antigen-specific T cells, and exceeded those for the negative control tetramer for all cohorts ($P<0.001$).

Samples for tetramer analysis were available for 10 patients before, during, and after treatment, for 14 patients from 2 time points, and for 2 patients at a single time point. Overall there were 19 lymphocyte samples before RT, 21 during RT and 20 after RT (Table 1).

Samples from four of 9 (44%) CRC patients before CRT treatment were positive for surviving tetramer binding (>2SD from the mean of healthy controls), 5 of 10 (50%) during treatment, and 8 of 12 (67%) after treatment (Table 1). This trend towards increased responses upon completion of CRT in CRC patients ($p=0.499$) was not apparent in prostate cancer patients ($p=0.352$) who exceeded the criterion of positivity in 5 of 10 (50%) cases before RT, 4 of 11 (36%) during RT and 4 of 8 (50%) after RT.

(place table 1 here)

3.2 Treatment-dependent Responses to Survivin

Because samples were taken before, during, and after treatment, we were able to evaluate individual patient responses over time (Figure 1A). The percent survivin-specific $CD8^+$ T cells increased in 9 out of 13 (69%) CRC patients as a result of treatment, including *before* → *during* RT (2 of 5 cases), *during* → *after* RT (4 of 7) and *before* → *after* RT (5 of 7). Statistical significance was not reached (p=0.267), but in only 2 of 13 cases (15%) was there a clear decrease in survivin-specific $CD8^+$ T cells after CRT. 7 out of 11 (64%, p=0.599) prostate cancer patients also responded to RT with an increase in survivin-specific CD8+ T cells, including *before* → *during* RT (3 of 10 cases), *during* → *after* RT (5 of 8 cases) and *before* → *after* RT (5 of 7 cases). Hence, CRT of colorectal cancer and RT of prostate cancer generally increased the percent of circulating survivin-specific T cells in the $CD8^+$ pool (P=0.076).

Interestingly, the ratio of $CD4^+$ to $CD8^+$ T cells increased in most patients from before to after completion of therapy (Figure 1B, $P_{CRCpatients}$=0.071 and $P_{prostate}$=0.47). This was most marked for CRC patients, who had a low CD4:CD8 ratio before therapy compared to all other cohorts (p=0.008 compared to healthy volunteers). Overall, cancer patients tended to have less $CD4^+$ in their circulation than healthy control subjects (33.6%±6.5) at the beginning of therapy (supplementary table).

3.3 Time course of response of circulating $T_{regulatory}$ cells

The number of $T_{regulatory}$ cells varied in 8 healthy volunteers (aged 30-64) from 1%-4.6% of total $CD4^+$ (mean 2.8±1.1% $CD4^+CD25^{high}Foxp3^+$) cells, which

compares well with the literature, indirectly validating our protocol. Most patients started treatment with less $T_{regulatory}$ cells in their circulation than the volunteers (Figure 2A, supplementary table). This was true for 7 of 8 (88%, p=0.182) CRC patients and for 13 of 17 (76%, p=0.083) prostate cancer patients. All 7 CRC patients with matched samples from before and after treatment ended the study with more $T_{regulatory}$ cells than they had initially, hence there was an overall trend for these values to increase (p=0.039, Figure 2B). Notably, the majority of prostate cancer patients (8 out of 13) did not adhere to this trend (Figure 2B).

3.4 Intratumoral Survivin expression

Tumor biopsies and resections from patients who were part of the CRC trial were stained with an antibody that recognizes both cytoplasmic and nuclear survivin. Examples are shown in Figure 3. All biopsies, with one exception, tested positive for cytoplasmic survivin, in most cases covering an extensive area of the tumor, whereas survivin was mostly undetectable in normal colon tissue, with the exception of crypts. Nuclear staining was seen more sporadically and in a smaller area of the tumor (Table 2). *(place table 2 here)*

3.5 Clinical responses

For patients in the clinical CRC trial, Table 2 summarizes the data on clinical tumor & lymph node stage and immunological parameters of survivin-specific CD8+T cells, $T_{regulatory}$ cells and intratumoral survivin expression. The patient numbers and subgroups were too small and the responses too variable to

derive statistical significance, but there were indications that a larger study could valuably explore.

Biopsy specimens from patients whose tumors were down-staged tended to have higher nuclear survivin levels at biopsy than those who were not down-staged (Figure 4A right panel). Both the ratio of nuclear to cytoplasmic survivin and staining intensity in biopsy specimens were higher for the responders (Figure 4B,C). The number of resection specimens was inevitably low, but both cytoplasmic and nuclear survivin were decreased over the comparable biopsy specimens, in particular for nuclear staining (Figure 4A,B). Only 6 patients who responded with T down-staging and 4 who did not respond could be evaluated for therapy-related changes in survivin-tetramer positive T cells, with the former showing more promising changes (Figure 4D).

4 Discussion

While reverse immunology has clearly established that survivin is immunogenic, it remains uncertain whether anti-survivin responses occur in untreated cancer patients. We were clearly able to detect T cells binding survivin tetramer in almost half of CRC and prostate patients prior to treatment when compared to healthy controls. This is in agreement with Coughlin et al. (40), who measured similar frequencies in pediatric cancer patients using the same tetramer, and Grube et al. (41) who detected survivin-reactive T cells in 40% of patients with multiple myeloma using IFNγ mRNA as a readout. It contrasts Casati et al. (17), who, using a different tetramer, reported that <0.1% of $CD8^+$ T cells from a CRC patient bound survivin, although this level could be boosted by in vitro stimulation.

From a phase I clinical vaccination trial we know that anti-survivin responses are not easily induced in CRC patients with only 1 of 15 responding (42). Assessing whether responses increase or decrease following RT or CRT alone therefore inevitably stretches the sensitivity and reproducibility of the assays being used. Nonetheless, we observed that tumor-specific T cells clearly increase in most CRC patients after completion of CRT and in most prostate cancer patients after RT. Perhaps more important is that only a few patients showed a decrease in survivin-reactive $CD8^+$ T cells, which suggests that their ability to respond is not compromised by treatment. This was also suggested in a randomized phase II clinical trial in prostate cancer patients where RT did not obstruct T cell responses to PSA when given at the end of a cancer vaccination

regimen (43).

It is tempting to ascribe the increase in tumor-specific T cells to a radiation-induced increase in antigenic peptide liberation (44) and presentation by dendritic cells, which we have shown can be boosted by radiation (45). The rate of tumor regression, i.e. tumor kinetics, may modulates this and hence the generation of immunity. The fact that this was not detected (43) by ELISPOT in patients with prostate cancer receiving RT could be due to the relative sensitivity of the assays. We have not been able to distinguish the two treatment arms of the CRC cohort with some patients receiving the COX-2 inhibitor Celecoxib because this trial is still ongoing. There are suggestions in the literature that Celecoxib might further enhance the development of antigen-specific $T_{effector}$ cells (46, 47), but this will not alter the conclusions from the study. The fact that CRC patients who responded with T down-staging tended to have higher levels of nuclear survivin suggests that this is a potential predictive marker of response, with the understanding that nuclear survivin relates more to proliferation and hence may signal a more rapid response (13, 48).

There are a number of ways to interpret radiation-induced anti-tumor reactive $CD8^+$ T cell levels. Loss of immune suppression due to decreased tumor burden is one. In recent years, $T_{regulatory}$ cells have gained prominence as a powerful suppressive mechanism. The frequency of $T_{regulatory}$ cells that we detected in the majority (80%) of our patients was actually lower than in healthy subjects, who were well within the published range for normal individuals (33, 49). This contrasts to several reports of high levels of circulating $T_{regulatory}$ cells in

cancer patients (33, 49, 50).

In our study, levels of $T_{regulatory}$ cells in CRC patients increased on completion of CRT while this did not happen in prostate cancer patients. It may be that in CRC $T_{regulatory}$ cells, and perhaps other $CD4^+$ T cell subsets, re-locate to tumor sites, for which there is evidence (31), and are remobilized by therapy-induced changes in the tumor microenvironment. RT-induced adhesion molecule and chemokine expression could alter migratory behavior of $T_{regulatory}$ cells (33, 51). It is also feasible that systemic effects induced by the CRT more so than by RT could have contributed to such a selective increase in $T_{regulatory}$ cells in CRC by affecting the balance in lymphocyte subpopulations (52). Such increases in circulating $T_{regulatory}$ cells may therefore be more apparent than real. Even local irradiation can affect the lymphocyte balance because lymphocyte subsets have differential sensitivity to radiation, with the simplified picture being that naïve T cells are more sensitive than effector cells (53), while antigen-induced $T_{regulatory}$ cells gain in radiosensitivity (36). The fact that we observed an increase in $CD4^+CD25^{high}Foxp3^+$ Tregs might simply reflect changes in other $CD4^+$ subpopulations.

Perhaps the most important point from this study is that CRT and RT do not induce immune tolerance to survivin making immunotherapy approaches feasible in combination with RT. Furthermore, tetramer technology coupled with other flow-based methods provide us with powerful tools to study the anti-tumor immune status in patients undergoing such treatments.

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LEGENDS

SUPPLEMENTARY FIGURE: Survivin tetramer binding in human PBMCs from patients and volunteers. The percent of $CD8^+$ cells in human PBMC samples binding the tetramer for survivin was higher when compared to the irrelevant, negative tetramer and higher in cancer patients than in 5 healthy volunteers (* $p<0.001$).

FIGURE 1: **A.** Levels of circulating survivin-reactive $CD8^+$ T cells for individual CRC (left) and prostate cancer (right) patients before, during, and after treatment. Solid lines indicate an overall upward trend, dashed lines a downward trend. **B)** Ratios of $CD4^+/CD8^+$ increase in patients before, during, and after treatment. Box and whisker diagrams summarize individual $CD4^+$: $CD8^+$ T cell ratios in cancer patients and in 5 healthy controls (* $p=0.008$).

FIGURE 2: The frequencies of circulating $T_{regulatory}$ cells in CRC and prostate cancer patients were generally less than those observed in 8 healthy volunteers and appeared to rise towards completion of CRT in colorectal cancer patients but not after RT in prostate cancer patients. Data are the % of $CD4^+$ cells that are $CD25^{high}$ and $Foxp3^+$ presented as **A)** box and whisker diagram and **B)** as time course for individual patients with solid lines indicating an upward trend (* $p=0.039$), dashed lines do not.

FIGURE 3: The tumor antigen survivin was highly expressed in CRC patient specimens, while it remained mostly undetectable in normal colon tissue. Survivin appeared to be primarily located in the cytoplasm but occasionally nuclear staining was evident [Magnification x800].

FIGURE 4: The expression of survivin in CRC tissue sections (21 biopsies, 10 resections). Responders (T down-staging) had higher nuclear survivin levels at biopsy and less at resection. Cytoplasmic surviving levels also were lower in resection specimens from responders. **(A)** Box and whisker diagram showing data as median percent tumor staining positive for cytoplasmic (left) and nuclear (right) survivin. Biopsies of patients who responded to pre-op CRT had **(B)** higher ratios of nuclear:cytoplasmic survivin, **(C)** express more survivin and **(D)** have more promising changes in circulating survivin-specific $CD8^+$ T cells than biopsies from non-responders. Solid lines indicate an upward trend, dashed lines do not.

TABLE 1: Increasing number of CRC patients show survivin-reactive CD8+ T cells in peripheral blood upon completion of radiation treatment. Data are from 15 HLA-A2 positive patients with colorectal cancer and 11 patients with prostate cancer. CRC indicates patients that were part of a COX II inhibitor clinical trial. Data are $CD8^+$ T cells staining positive with the tetramer for survivin [%] and levels of $CD8^+$ PBMCs [%]. Two batches of survivin tetramer were used and are indicated. 5 healthy volunteers served as control. Gray fields highlight those samples that stained above the background level (=> mean ± 2x SD of % survivin-reactive $CD8^+$ T cells in the healthy volunteers for that tetramer batch).

	tetramer [% of CD8]				CD8 [% of PBMC]		
	before	**during**	**after**	**tet set**	**before**	**during**	**after**
CRC 7		0.04	0.09	1		14.9	14.1
CRC 19		0	0.18	1		3.2	5.4
CRC 21	0.1		0.16	1	15.5		1.6
CRC 22		0.76		2		1.2	
CRC 23	1.57	1.93		2	12.8	10.9	
CRC 24	1.16	0.27	0.97	2	5.78	15.5	12.1
CRC 27		1.67	0.75	2		1.9	4.8
CRC 28			0.42	2			9.4
CRC 29	0.68		2.25	2	18.1		24.5
CRC 30		1.48	1.46	2		7.24	10.1
CRC 33	0.12	0.17		2	28.2	20.8	
CRC 34	0.23	0.15	0.12	2	8.4	7.6	5.3
rectum 5	0.02		0.18	1	11.9		14.9
rectum 8	0.04	0.03	0.07	1	14.7	11.5	10.5
rectum 10	0.01		0.07	1	18.2		8.95
prostate 05	0.1	0.11		1	15.2	21.6	
prostate 1	0.23	0.29	0.37	2	16.4	13.7	13.8
prostate 2	0.12	0.05	0.25	2	17	18.7	17.7
prostate 3	0.28	0.23	0.53	2	4	4.1	3.3
prostate 4	1.33	0.16		2	17.7	18.5	
prostate 5	0.22	0.12		2	8.9	8.1	
prostate 6	0.15	0.15	0.26	2	13.9	15.7	7.3
prostate 9	0.46	0.16	0.15	2	5.4	7.7	9
prostate 11	0.32	0.31	0.21	2	28	40	36
prostate 12	0.15	0.5	0.32	2	6	2.5	5.5
prostate 13		0.1	0.14	2		5.4	5.5

TABLE 2: Summarized data from 30 CRC patients that were part of the clinical trial. "1" is a positive indicator for the respective parameter whereas "0" specifies a negative response. Tumor (n=21) and lymph node (n=22) stage decreased or did not (n=9 and n=5, respectively). 12 Patients were HLA-A2 positive. The number of survivin-reactive $CD8^+$ T cells increased in the peripheral blood in 4 of 6 responding patients and 2 of 4 non-responders. The frequency of systemic Treg cells increased in 6 of 7 responding patients and 4 of 4 non-responders. All biopsies tested stained positive for survivin, which was mostly in the cytoplasm and lesser so in the nucleus. In most samples, nuclear but not cytoplasmic surviving staining decreased and in all resection specimens from the 3 responders was essentially zero.

% cytopl.= percent tumor staining positive for survivin in cytoplasm, intens. cyto = intensity of the cytoplasmic staining, % nuclear = percent tumor staining positive for survivin in the nucleus.

CRC	T-	N-	HLA-A2	tetramer	Treg	survivin expression in biopsy			survivin expression in resect		
patient	downstaging	downstaging		increasing	increasing	% cyto.	intens. cyto.	% nuclear	% cyto.	intens. cyto.	% nuclear
11	0	0				74	3	14	52.5	2	3.75
12	0	1	0		1	80	1	10			
16	0	1	0			90	2	0	100	3	5
17	0	0				73.3	1	3.3			
19	0	N/A	1	1		77.5	2	3			
24	0	1	1	0	1				92	2	24
26	0	1	0		1	0	0	20	34.2	1	1.4
27	0	0	1	0	N/A	58	1	0	77	3	7
33	0	1	1	1	1						
1	1	1	0			66.7	2	6.7			
2	1	1	0			70	2	7.5			
3	1	1				74	2	12			
4	1	0				90	3	2			
7	1	1	1	1		50	1	N/A	55.4	2	0.38
8	1	N/A			N/A	88	3	1			
9	1	1				68.3	3	28.3			
10	1	1				88	2	40			
18	1	N/A				70	3	1.25			
20	1	1	0			32.8	2	37.14	100	3	0
21	1	0	1	1		65	1	2.5			
22	1	1	1	N/A							
23	1	1	1	1					30	1	0
28	1	1	1	N/A	N/A	10	1	40			
29	1	1	1	1	1	40	1	0			
30	1	1	1	0	1	75	2	0			
31	1	1	0		1						
32	1	1	0		1						
34	1	1	1	0	0						
35	1	1	0		1						
36	1	1	0		1						

SUPPLEMENTARY TABLE: The frequency of circulating $T_{regulatory}$ cells in cancer patients were generally less than those observed in 8 healthy volunteers (2.8 ±1.1) but appeared to raise towards completion of cancer-radiotherapy and thereafter in colorectal cancer patients more so than in prostate cancer patients. Data are the % of $CD4^+$ cells that are $CD25^{high}$ and $Foxp3^+$. CRC indicates colorectal cancer patients that were part of a COX II inhibitor clinical trial. Levels of $CD4^+$ PBMCs were also reduced these cancer patients compared to healthy controls (34% ± 6.5%).

	Treg [% of CD4]			CD4 [% of PBMC]		
	before	during	after	before	during	after
CRC 8		5.8			8	
CRC 12	2.3		13	9		26
CRC 20		0.8	3.7		24	4
CRC 24		1	1.3		26	16
CRC 26	1.2	2.4	1.4	38	46	39
CRC 27			4			16
CRC 28			4.4			38
CRC 29	2.3		3.9	14		20
CRC 30		2.1	2.7		38	28
CRC 31	3	4.7	3.5	22	24	9
CRC 32	1.6	1.8	1.7	18	13	9
CRC 33	1.4	1.6		10	26	
CRC 34		1.3	0.4		33	31
CRC 35	2.1	4.6	4.1	40	41	24
CRC 36	2.6		5.6	17		11
prostate 03	4.3		3.4	28.5		37.7
prostate 04	0.7	0.7	0.6	37	39.8	30.4
prostate 05	0.4			34		
prostate 06	0.4		0.1	45		53
prostate 07	3.6		3.8	19		13
prostate 08	2.5	1.6	2.5	54	47	49
prostate 1	2.5	2	2.3	23	18	24
prostate 2	2.2	3.1	3.3	28	19	20
prostate 3	1.1	2.9	2.2	22	14	11
prostaet 4	1.8	2.3		19	24	
prostate 5	2.8	2.5	3.3	32	25	12
prostate 6	4.7			20		
prostate 7	2.5	1	2.2	34	16	9
prostate 8	1	0.8	1.6	14	13	20.4
prostate 9			0.5			35
prostate 10	1.2	0.8		24	19	
prostate 11	2.5	0.5	0.7	17	18	22
prostate 12	1.1		0.9	14		19
prostate 13		1.5	0.7		36	34